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<b>(21) International Application Number:</b> PCT/GB95/00208 <b>(22) International Filing Date:</b> 2 February 1995 (02.02.95) <b>(30) Priority Data:</b> 9401987.4                      2 February 1994 (02.02.94)                      GB <b>(71) Applicant (for all designated States except US):</b> IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE [GB/GB]; Sherfield Building, Imperial College, London SW7 2AZ (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KARAYIANNIS, Peter [GB/GB]; 54 Townsend Avenue, London N14 7HJ (GB). THOMAS, Howard, Christopher [GB/GB]; 39 Beech Drive, London N2 9NX (GB). <b>(74) Agents:</b> SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).		<b>(81) Designated States:</b> AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> HEPATITIS B VACCINE <b>(57) Abstract</b> <p>A variant and so-called "escape mutant" HBsAg protein or fragment thereof displaying the antigenicity of hepatitis B virus surface antigen is disclosed, in which the mutant protein or fragment thereof (mHBsAg) comprises a modified 'a' determinant in which at least two amino acids are inserted downstream of position (122) of the wild type HBsAg sequence. A vaccine comprising the mHBsAg is provided, as is a kit for diagnostic <i>in vitro</i> detection of anti-mHBsAg antibodies and an antibody preparation comprising anti-mHBsAg antibodies.</p>		

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HEPATITIS B VACCINE

This invention relates to recombinant DNA molecules which code for polypeptides having specificity towards a hepatitis B viral antigen.

More particularly this invention relates to a vaccine composition for stimulating the production of antibodies in humans to a variant hepatitis B virus.

The cloning of the genomes of hepatitis B virions of different serotypes is well known in the art (Miller et al). Dane particles which are hepatitis B virions and are isolatable from infected patients have a diameter of approximately 42nm. Each consists of an envelope comprising the hepatitis B surface antigen (HBsAg), a capsid (HBcAg), an endogenous polymerase and a DNA genome. A third polypeptide, 'e' antigen (HBeAg) is made by the hepatitis B virus and found in solubilized form in serum.

Infection with hepatitis B virus (HBV) is a serious, widespread problem but vaccines for use in mass immunisation are now widely available. Vaccines commercially available against HBV comprise hepatitis B virus surface antigen (HBsAg) either in native or recombinant form. The authentic (or wild type) hepatitis B virus surface antigen can be recovered from plasma of infected individuals as a particle of about 22nm comprising of two proteins known as P24 and its glycosylated derivative GP28, both of which are encoded by a 226 amino acid coding sequence on the HBV genome known as the S protein coding sequence or HBV S-gene (Tiollais et al., (1985)). The complete amino acid

sequence as well as the nucleotide sequence encoding, HBsAg is given in Valenzuela et al., *Nature* 280 815 (1979). The numbering system used by Tiollais et al. to define nucleotide and amino acid positions is used  
5 herein.

Insertion of HBV S-gene coding sequences under the control of yeast promoters on expression vectors to enable expression of HBsAg in *S. cerevisiae* for vaccine  
10 production has been described by Harford et al. in *Develop. Biol. Standard* 54 125 (1983), Valenzuela et al., *Nature* 298 347 (1982) and Bitter et al., *J. Med. Virol.* 25 123 (1988). Expression in *Pichia pastoris* has been described by Gregg et al., *Biotechnology* 5 479 (1987),  
15 (see also European Patent Publication No. 0226846) as has expression in *Hansenula polymorpha* (European Publication No. 0299108).

Vaccines have also been prepared from hybrid immunogenic  
20 particles comprising HBsAg protein as described in European Patent Publication No. 0278940. Such immunogenic particles can contain, for example, all or parts of the HBsAg precursor protein encoded by the coding sequence which immediately precedes the HBV-S gene  
25 on the HBV genome, referred to herein as the Pre-S coding sequence. The Pre-S coding sequence normally codes for 163 amino acids (in the case of the ay HBV sub type) and comprises a Pre-S1 coding sequence and a Pre-S2 coding sequence. The latter codes for 55 amino acids and  
30 immediately precedes the S protein coding sequence (European Publication No. EP-A-0278940).

The surface antigen (the S antigen) open reading frame of HBV-DNA is divided into three regions, pre-S1, pre S-2

and S. It encodes three envelope proteins of HBV termed: large, middle and major proteins. The major protein, HBsAg, consists of 226 amino acids and is encoded by the S gene (Tiollais et al., (1981); Tiollais et al., (1987) and Lau et al.). All three envelope proteins contain HBsAg antigenic sites and are easily detected by conventional immunoassays for HBsAg. These immunoassays are extensively used for diagnosing HBV infection and screening blood donors world-wide. The HBsAg reactivity is dependent on the structural conformation of the hydrophilic region from amino acids 124-147 which is defined as the 'a' determinant (Ashton-Rickardt et al. and Brown et al.). This is common to all HBV subtypes and antibody to it confers protection against reinfection with any of the subtypes.

HBV-DNA sequences hybridising under highly stringent conditions with an HBV probe have been shown in the liver, serum and blood mononuclear cells of subjects negative for serum HBsAg (Brechot et al. (1985) and Thier et al). Recent results from different laboratories using dot blot hybridisation or Polymerase Chain Reaction (PCR) confirm the presence of HBV DNA sequences in serum from HBsAg negative subjects. The development of PCR techniques has permitted the detection of very low levels of HBV replication in many patients and has allowed sequencing of many isolates which have identified genetic variation in some isolates of the virus (Carman et al., (1990); Brechot et al., (1991); Blum et al.). In Taiwan and Sardinia, where HBV is highly endemic, 1.7% and 0.3% of HBsAg negative healthy blood donors had HBV DNA in their sera detectable by dot blot hybridisation (Lai et al., (1989) and Sun et al.) In mainland China, a molecular epidemiological investigation using PCR in

anti-HB-positive individuals indicated the existence of HBV carriers with undetectable HBsAg, which accounts for 3% of the Chinese general population (Luo et al.)

5 Antigenic subtypes of HBV are defined serologically and have been shown to be caused by a single base changes in the region of the genome encoding HBsAg (Okamoto et al.). However, all presently known antigenic subtypes contain the 'a' determinant consisting of amino acids 124 to 147  
10 of HBsAg. Antibodies to the 'a' determinant confers protection against all subtypes. It has been shown by in vitro mutagenesis that the cysteine at position 147 and the proline at position 142 are important for the exhibition of full antigenicity of the 'a' determinant  
15 (Ashton-Rickardt et al.).

Additionally, Howard Thomas and William Carman, detailed in WO 91/14703 a variant of an HBsAg fragment in a vaccinated child born to an HBV infected mother.  
20 Sequencing revealed a point mutation from guanosine to adenosine at nucleotide position 587 resulting in an amino acid change from glycine to arginine at position 145 in the 'a' determinant of HBsAg (see also Carman et al., (1990)). Similar HBV mutants have been reported  
25 to replicate in the host under humoral immune pressure, either actively (vaccine) or passively (hyperimmune globulin) induced (Okamoto et al., (1992) and McMahon et al.) However, the persistent presence of both HBsAg and anti-HBs (vaccine induced) detected by conventional  
30 immunoassays, in the serum of some of these patients, suggests that the mutation does not result in complete loss of antigenicity (Carman et al., (1990), Okamoto et al., (1992) and McMahon et al.).

From the clinical and epidemiological view, it is more important to investigate the molecular features of HBV from subjects without any HBsAg reacting in standard assays. Sequencing results from a Japanese patient who was HBeAg and HBV-DNA, as well as anti-HBs positive showed that amino acids 9 to 22 of the pre-S2 region were deleted whereas amino acids at position 3 and 8 of the pre-S2, and 126, 131 and 133 of the first loop of the 'a' determinant of HBs were substituted (Moriyama et al.). Analysis of the deduced amino acid sequence from another isolate revealed substitutions at positions 3, 53 and 210 of the major HBs protein, all of which are outside the common 'a' determinant and cannot really explain the absence of HBsAg in serum (Liang et al.).

During the last decade, several putative variants or mutants of hepatitis B virus have been described. For instance, McMahon et al. have described a substitution of arginine for glycine in a putative monoclonal antibody binding domain of HBsAg (as deduced by DNA sequence analysis) in a liver transplant patient treated with anti-HBsAg monoclonal antibody (Cold Spring Harbor Symposium on the Molecular Biology of Hepatitis B Viruses, September, 1989).

In an investigation by Carman et al. and the subject of patent application WO 94/26904, a mutant hepatitis B virus having a modified 'a' determinant wherein two specific amino acids, asparagine and threonine, were inserted at position 122 of the HBsAg sequence. Further, a mutation at position 145, substituting glycine for the wild type arginine was also detailed in the amino acid sequence listing.

In another report, children and adults were found with circulating hepatitis B surface antigen, indicating viral replication, despite the presence of specific antibody (anti-HBs) after immunisation with one of two licensed hepatitis B vaccines (Zanetti et al.). Analysis of the HBsAg with monoclonal antibodies revealed that the circulating antigen did not carry the 'a' determinant or that this determinant was masked. It was concluded that emergence of a variant of hepatitis B virus had been detected, possibly due to epidemiological pressure associated with immunisation in an endemic area of infection. The variant was, however, not characterised further.

From the work of Zanetti et al. it is clear that a great disadvantage with presently available hepatitis B vaccines is that they may, at least in a host with a predisposing immunogenetic make-up, cause the appearance of an 'escape mutant', i.e. a replicating infectious virus that has mutated away from neutralising immunity. Such a variant virus obviously has the capacity to cause disease and may be assumed to be transmissible. The variant virus may therefore give rise to a serious immunisation problem since it is not effectively neutralised by antibodies produced by vaccines based on normal HBsAg. Other mutations have been described in HBV, but their significance in terms of altered antigenicity is unclear (Moriyama et al. and Lai et al., (1990)).

In China, a highly endemic area for HBV infection, recent studies, utilising PCR, suggest that 30-40% of HBsAg negative patients with either cryptogenic cirrhosis, chronic active hepatitis (CAH) or chronic persistent



hepatitis (CPH), have replicating HBV-DNA in serum or liver tissue (Zhang et al.). These studies show, that a variant of HBV exists in the Chinese population, characterised by undetectable HBsAg in serum. Surprisingly, such a putative mutant has never been characterised at the molecular level.

The isolates from our Chinese patients (i.e. patients without any detectable HBsAg) are insertion mutants where additional amino acids are inserted between codons 122 and 124, immediately before the 'a' determinant. This sequence variation has not previously been described in any known HBV subtype and was not seen in 30 HBsAg positive Chinese patients from the same region. It is interesting to note that the region in which the insertion occurred is an important epitope area of HBsAg. The inserted sequence is found immediately downstream of codon 122 which determines the subtype determinant (d) and immediately upstream of the 'a' determinant. All nucleotide and amino acid sequences before and after codon position 122 are as detailed in wild type HBV (Figures 6 and 7). Without wishing to be bound by theory, we believe this mutation results in a conformational change affecting epitopes of the 'a' determinant and of the 'd' subtype. One likely possibility is that the insertion of the amino acids changes the epitopes by affecting the spatial organisation of the two loops of the 'a' determinant. Another possibility is that the first loop of the 'a' determinant, defined by monoclonal antibodies (Waters et al.), may include more upstream amino acids (than previously thought). We propose that these amino acids are being formed by a disulphide bridge upstream of 122 rather than 124 as previously suggested (Waters et al.

and Ashton-Rickardt et al.). The inserted amino acids would increase the span of the loop from 14 to 16 or 17 amino acids therefore altering the conformation of this loop, preventing binding of neutralising antibody.

5

In this invention, we describe a new variant or 'escape mutant' of HBV isolated from Chinese patients whose serum was positive for HBV-DNA by dot blot hybridisation but HBsAg negative by commercial polyclonal antibody based immunoassays. Additionally, the present invention overcomes, or at least mitigates, the disadvantages associated with known HBV vaccines as these vaccines are ineffective for these newly discovered mutants.

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The present invention provides characterisation of newly ascertained mutants of HBV that have at least two amino acid insertions immediately downstream of position 122 at the HBV envelope region. The present invention provides methods for determining the presence of the mutant HBV in a test sample, and reagents useful in these methods. All aspects of this invention provide a modification of the 'a' determinant in which there is an insertion of at least two amino acids downstream of position 122 of the HBsAg sequence, which corresponds to at least a six nucleotide insertion downstream of nucleotide 519 of the HBsAg genome.

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The nucleic acid sequence derived from mutant HBV, or a portion thereof are useful as probes to determine the presence of mutant HBV in test samples. The sequence also makes available polypeptide sequences of mutant HBV antigen(s) encoded within the genome(s) of such mutant HBV and permits the production of polypeptides which are useful as standards or reagent sin diagnostic tests

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and/or as components of vaccines. Monoclonal and polyclonal antibodies directed against an epitope contained within these polypeptide sequences, also are useful for diagnostic tests as well as therapeutic agents, for screening of antiviral agents, and for the isolation of the mutant HBV from which these nucleic acid sequences are derived.

It is to be understood that this mutant HBsAg may also include Pre-S sequences if so desired.

The variant HBsAg protein or fragment thereof according to the invention is hereinafter abbreviated "mHBsAg".

It will be appreciated that these mHBsAg mutants are not in a 'naturally occurring' form but are synthetic or a highly purified material, free of blood products.

Preferably these mutants as described in the invention correspond to full length HBsAg and are identical to wild type HBsAg except for the insertion of at least two amino acid residues downstream of position 122.

Preferably the HBsAg mutants are in a highly purified form, for example in a state of purity greater than 75%, more preferably greater than 90%, and most preferably 95-100% pure.

In a further aspect of the present invention there is provided a vaccine composition comprising an immunoprotective amount of these HBsAg "escape mutants" combined with a suitable carrier.

Other aspects of the invention are described hereinbelow.

5 The mHBsAg and vaccine of the invention may be used to overcome the problems perceived by the emergence of an 'escape mutant' as defined hereinabove in which the area immediately upstream of 'a' determinant (the envelope region) of the viral HBsAg has undergone modification. In particular, the vaccine of the invention has the advantage in that it may be used to protect against, and  
10 prevent the emergence or transmission of, a variant HBV which is defined herein as having a modified HBV envelope region in the HBsAg amino acid sequence wherein there are at least two amino acids inserted downstream of position 122.

15 Accordingly there is also provided a method for protecting a human against disease symptoms associated with infection with said variant HBV, which method comprises administering to the human a safe and effective  
20 amount of the vaccine according to the invention.

In another aspect the present invention provides mHBsAg for use in therapy, especially prophylaxis.

25 The invention also provides the use of mHBsAg in the manufacture of a vaccine composition for protecting a human against disease symptoms associated with said variant HBV infection.

30 When used to immunise humans against an existing variant HBV virus it will be appreciated that the mHBsAg sequence in the vaccine will normally match, or be antigenically equivalent to, the mHBsAg sequence in the variant HBV virus.

Preferably the amino acid residues inserted after position 122 in the mHBsAg of the invention is such that it may be derived by an insertion of six or more nucleotides. The inserted nucleotides encoding the extra amino acids may affect any of the 3 nucleotides of codon 123 in normal HBsAg.

Theoretically, any mutation which changes the hydrophilicity of the envelope proteins can result in altering antigenicity and the appearance of variant HBV. The hydrophilicity of the wild type and that of the mutant in this region are quite different. A reduction in hydrophilicity described here is often correlated with a loss of antigenicity. The increased hydrophobicity of the mutant in the first loop may also result in an altered protein of the major protein in the lipid of the envelope thereby having a profound effect on the conformation.

In a preferred embodiment of the invention, the residues after position 122 of normal HBsAg may reduce the hydrophilicity of the 'a' determinant.

In another preferred embodiment of the invention the mHBsAg is identical to normal (wild type) HBsAg S-protein except for the insertion of at least two amino acid residues downstream of position 122.

In a particularly preferred embodiment of the invention the modification downstream of position 122, is an insertion of arginine and then alanine, respectively. Also preferably, an insertion downstream of position 122 of arginine, glycine and alanine in that order may be inserted.

Preferred features of each aspect of the invention are as for each other aspect, *mutatis mutandis*.

5 The invention will now be illustrated by the following examples. The examples refer to the accompanying drawings, in which:

10 FIGURE 1 illustrates serial serum aminotransferase (ALT) levels from Patient No. 1. Normal levels are below 40 i $\mu$ /L. Serum HBV markers were measured at various prints in time. In August 1992 HBV DNA was found by PCR yet HBsAg and the other HBV markers were undetected.

15 FIGURE 2 illustrates serial serum ALT levels from Patient No. 2 as well as serum hepatitis B virus markers. Abbreviations indicate: w, wild type; m, mutant.

20 FIGURE 3 is a diagrammatic representation of the surface gene open reading frame, position of the PCR and sequencing primers, and the overlapping polymerase open reading frame (P ORF). Shaded circles represent initiation and stop codons,  
25 whereas the shaded box indicates the insertion hot spot in relation to the 'a' determinant (positions 522-593). The nucleotide numbering is from a hypothetical EcoRI site as in published HBV DNA sequences, because the present isolates did not  
30 possess an EcoRI site.

FIGURE 4 is an autoradiograph showing the nucleotide insertion in Isolates 1 and 2 (left and right) compared with the wild type (centre) following

direct sequencing of PCR products. The arrows indicate the point of insertion.

5       FIGURE 5 illustrates the nucleotide and amino acid sequences of Isolates 1 and 2 compared with wild type (adw). The inserted sequences are underlined.

10       FIGURE 6 illustrates the complete nucleotide sequence of mHBsAg Isolate 1. The top sequence represents that of the surface gene and 'a' determinant the mutant HBsAg isolate whereas the bottom sequence represents the wild type sequence. (---) represent points of amino acid insertion.

15       FIGURE 7 illustrates the complete nucleotide sequence of mHBsAg Isolate 2. The top sequence represents that of the surface gene and 'a' determinant the mutant HBsAg isolate whereas the bottom sequence represents the wild type sequence.  
20       (---) represent points of amino acid insertion.

Table 1 depicts the synthetic oligonucleotide primers used for the Polymerase Chain and sequencing reactions.

25       Table 2 illustrates positive/negative binding ratios of serum HBsAg from Patient Nos. 1 and 2 to monoclonal antibodies with adw wild type used as a control.

30       Table 3 sets out the nucleotide and amino acid sequence homologies of mutant S genes compared with previous published wild type sequence (adw) and a Chinese isolate from the same region as the wild type.

HBV mutations are more commonly found in particular areas of the sequence characterised by runs of bases. In the case under study the insertion is associated with a run of four adenines. This area is seemingly a "hot spot" for insertion in some isolates from Chinese HBsAg negative HBV carriers.

In a further aspect of the invention there is provided a process for preparing the mHBsAg and the vaccine composition obtained therefrom.

Preferably the mHBsAg is obtained synthetically, either by peptide synthesis or more preferably by recombinant DNA techniques.

Methods for the construction, manipulation and verification of recombinant DNA molecules and sequences are well known in the art. To modify the HBV S protein of HBsAg and obtain the mHBsAg of the invention it is desirable to insert the codons CGG and GCA, or any other triplet codon combination which encodes arginine and alanine, respectively, downstream of position 122. Alternatively, codons CAC, GGG or GCG, or any other triplet codon combination encoding arginine, glycine and alanine, respectively, may be inserted between nucleotide positions 122 and 124.

Several methods are available to effect the appropriate change of sequence. One suitable method is complete *de novo* synthesis, by phosphite or phosphoramidite chemistry, of the desired coding sequence using viral or yeast codon frequencies.



Synthesis of DNA is available from several companies on a commercial basis. An example of such gene synthesis is described by Hayden and Mandecki, DNA 7: 571 (1988) and references therein.

5

A second method is to clone on a single strand vector an appropriate restriction fragment from a vector which already comprises the HBV genome and thereafter effect site specific *in vitro* mutagenesis as described by Botstein and Shortle, *Science* 229 1193 (1982). A culture of *E. coli* K12 strain C600 containing the recombinant plasmid pRIT10601 comprising an HBV genome of ay subtype cloned on pBR322 was deposited in accordance with the Budapest Treaty in the American Type Culture Collection on 2 June 1982 under Accession Number ATCC 39132. The sequence coding for the S-gene specifying the 226 amino acid HBsAg protein or longer sequences coding for Pre S polypeptides can be excised from such clones by standard recombinant DNA techniques.

20

One appropriate restriction fragment is the 575 bp *Xba*I-*Acc*I fragment from within the S-gene coding region of pRIT10601. Vector systems useful for *in vitro* mutagenesis are commercially available. The mutated gene fragment so obtained is reinserted into the S-gene.

25

A third method is to effect the desired mutational change using polymerase chain reaction (PCR) technology as described by Ho et al., *Gene* 77 51 (1989).

30

In each case the mHBsAg coding sequence may be expressed under the control of a suitable promoter in any suitable host.

Expression vectors comprising the DNA sequence encoding mHBsAg are novel and form a further aspect of the present invention. Hosts transformed with the said expression vectors form yet another aspect of the invention.

5

In a preferred aspect *S. cerevisiae*, *Pichia pastoris* or *Hansenula polymorpha* may be used as the host and expression is under the control of a yeast promoter, such as the yeast TDH3 promoter (glyceraldehyde- 3-phosphate dehydrogenase gene, see Valenzuela et al., 1982; Bitter et al., 1988) or PH05 (Miyanochara et al., 1983), MOX, FMDH (see EP-A-0299108) and AOX (see EP-A-0226846).

10

The transformed host can be cultured or fermented by conventional means and the mHBsAg extracted and purified. Purification of HBsAg from yeast cells is well known in the art and can be done according to any of U.S. Patent Nos. 4,649,192; 4,683,294; 4,694,074 or 4,738,926. Purification of the mHBsAg of the invention is carried out in an analogous manner.

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Vaccines containing the mHBsAg are prepared by conventional techniques and will contain an immunoprotective amount of the mHBsAg preferably in buffered physiological saline and admixed or adsorbed with any of the various known adjuvants including aluminium hydroxide and aluminium phosphate. By "immunoprotective" is meant that enough of the mHBsAg is administered to elicit a sufficient protective antibody or cell mediated immune response to confer protection against the infectious agent without serious side effects. The amount of mHBsAg to be administered will depend on whether the vaccine is adjuvanted and will generally comprise between 1 to 1000 mcg of protein.

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Preferably 1 to 200 mcg protein is used or, more preferably 5 to 40 mcg protein. The amount and number of doses to be administered can be determined in standard dose range studies involving observation of antibody titres and other responses in subjects.

The mHBsAg may also be mixed with other HBsAg such as normal HBsAg or homogenous or composite HBsAg particles containing all or part or parts of the PreS1 or PreS2 polypeptides for vaccine formulation. It may also be mixed with hybrid HBsAg particles carrying epitopes from proteins from other organisms and with other immunogens to form bivalent or multivalent vaccines. Vaccine preparation is generally described in "Vaccines", edited by Voller et al., University Park Press, Baltimore, MD, U.S.A., 1978.

The mHBsAg is useful for inclusion as an immunological reagent in detection kits for variant HBV virus infection and the like. It can also be used to raise polyclonal and monoclonal antibodies by known methods, some of which monoclonal antibodies may be specific for the variant antigen and not recognise normal HBsAg.

In another embodiment of the invention polypeptides which react immunologically with serum containing mutant HBV antibodies and composites thereof, and the antibodies raised against the mutant HBV specific epitopes in these polypeptides are useful in immunoassays to detect the presence of mutant HBV antibodies, or the presence of the virus and/or viral antigens in biological test samples. The design of these immunoassays is subject to variation, and a variety of these are known in the art; a variety of these have been described herein. The immunoassay may

utilise one viral antigen, such as a polypeptide derived from any clone-containing mutant HBV nucleic acid sequence, or from the composite nucleic acid sequences derived from the mutant HBV nucleic acid sequences in these clones, or from the mutant HBV genome from which the nucleic acid sequences in these clones is derived. Or, the immunoassay may use a combination of viral antigens derived from these sources. It may use, for example, a monoclonal antibody directed against the same viral antigen, or polyclonal antibodies directed against different viral antigens. Assays can include but are not limited to those based on competition, direct reaction or sandwich-type assays. Assays may use solid phases or may be performed by immunoprecipitation or any other methods which do not utilise solid phases. Examples of assays which utilise labels as the signal generating compound and those labels are described herein. Signals also may be amplified by using biotin and avidin, enzyme labels or biotin anti-biotin systems, such as that described in pending U.S. patent application Serial Nos. 608,849; 070,647; 418,981; and 687,785. Recombinant polypeptides which include epitopes from immunodominant regions of mutant HBV may be useful for the detection of viral antibodies in biological test samples of infected individuals. It also is contemplated that antibodies may be useful in discriminating acute from non-acute infections. Kits suitable for immunodiagnosis and containing the appropriate reagents are constructed by packaging the appropriate materials, including the polypeptides of the invention containing mutant HBV epitopes or antibodies directed against mutant HBV epitopes in suitable containers, along with the remaining reagents and materials required for the conduct of the assay, as well as suitable assay instructions.

Accordingly in a preferred aspect of the invention there is provided a kit for the diagnostic in vitro detection of anti-mHBsAg antibodies in a biological medium, and in particular neutralising antibodies following vaccination, characterised in that it comprises:

- a) mHBsAg as herein defined; and
- b) means adapted to detect the antigen-antibody reaction.

In a further aspect the invention provides an antibody preparation comprising anti-mHBsAg antibodies for use in the diagnosis, prevention or treatment of hepatitis B infection in humans. There is also provided a method of treating humans with an effective amount of such anti-mHBsAg antibody to prevent or treat hepatitis B infection.

The invention will now be illustrated by the following example.

#### EXAMPLE

Patient No. 1 was a 58 year old male, with a 6 year history of non-A, non-B chronic hepatitis. In August 1992, HBV-DNA was found by PCR in the absence of HBsAg and other HBV markers (Fig. 1). At this stage, the patient had cirrhosis.

Patient No. 2 was a 23 year old woman from the south of China, who on routine testing in March 1993 had a slightly elevated serum aminotransferase (ALT) level and was positive for HBsAg and HBeAg, but negative for both immunoglobulin (Ig)M and IgG anti-HBs. On follow-up in

January 1994, she continued to be HBV-DNA positive and was now also anti-HB positive but negative for HBsAg, HBeAg and anti-HBs (Fig. 2)

- 5 Both patients were negative for hepatitis C virus (HCV) and HCV-RNA. Thirty HBsAg positive Chinese patients with chronic liver disease or hepatocellular carcinoma were studied from the same region.

10 **Serological Investigation**

- HBsAg, HBeAg, total anti-HBc, IgM anti-HBs and anti-HBs were tested by commercially available enzyme immunoassays (Abbott Laboratories, North Chicago, Illinois, U.S.A.). HBsAg and anti-HBs results were confirmed in the United  
15 Kingdom.

**Monoclonal Antibody Binding**

- A panel of anti-HBs monoclonal antibodies known to bind to the 'a' determinant, were used to define more  
20 specifically changes in the 'a' determinant epitopes. Polystyrene beads (Northumbria Biologicals, Northumberland, UK) were coated with antibodies RFHBs-1, RFHBs-2 and RFHBs-7, and then incubated with the serum samples diluted 1:2 in 50% newborn-calf serum in  
25 phosphate-buffered saline, pH 7.2. Bound HBsAg was detected with  $^{125}\text{I}$ -anti-HBs from an 'AusRIA II' kit (Abbott Diagnostics), RFHBs-1 and RFHBs-2 bind to a cyclical peptide containing the sequence amino acids 124-137 and RFHBs-7 to a cyclical peptide from amino-acids  
30 139-147 of HBsAg.

**Hepatitis Markers**

- HBsAg, HBeAg, total anti-HBc, IgM anti-HBc, and anti-HBs were tested by enzyme immunoassay (Abbott Laboratories,  
35 North Chicago, IL).

#### Dot Blot Hybridisation

HBV-DNA was detected by dot blot hybridisation using a modification of a method detailed in Weller et al *J. Med. Virol* 9 273-280 (1982).

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#### Extraction of HBV-DNA, PCR and Direct Sequencing

50ul of serum were digested in the presence of 0.5% sodium dodecyl sulphate, 25mM sodium acetate, 2.5mM EDTA, 1mg/ml proteinase k (Boehringer Mannheim, Lewes, UK) in a volume of 200μl for 2 hours at 68°C, two phenol-chloroform extractions were followed by two chloroform extractions and HBV-DNA was precipitated with ethanol. After washing twice with 70% ethanol, the DNA pellets were resuspended in 10ul of water.

15

Five μL of the extracted HBV-DNA was used as template for PCR amplification. One set of primers (adw subtype), PO5' (5'-TGCGGGTCACCATAT, position 2818-2833), and POL3 (5'-AAGGATCCAGTTGGC, position 1409-1395) (Table 2) were used to get a 1800bp fragment which included the pre-S1, pre-S2 and S genes (Figure 3). Another set of primers, M3 (5'-CTGGGAGGAGTTGGGGAGGAGATT, position 1781-1755) and 3C (5'-CTAACATTGAGATTCCCGAGA, position 2460-2439) were used to amplify the Pre-C and C regions. The same serum sample was analysed independently in China and Britain using different sets of primers.

25

Direct sequencing of the PCR products was carried out with the 'fmol' Sequencing Kit (Promega Co, Southampton, England). Sequencing primers (Table 1) were end-labelled with 25μCi of <sup>32</sup>P-adenosine triphosphate (Amersham International, Amersham, UK) in a volume of 10μL using 10 units of polynucleotide kinase (Boehringer Mannheim) for 10 minutes at 37°C. 1.5μL of the reaction mix was

30

directly used in sequencing reactions. 90 $\mu$ L of PCR product were precipitated with an equal volume of 4 mol/L sodium acetate and 2 volumes of isopropanol for 10 minutes at room temperature, pelleted by centrifugation for 10 minutes, and then washed two times with 70% ethanol. After resuspension in 20 $\mu$ L of water, 9.5 $\mu$ L of DNA was added to the reaction buffer with the end-labelled primer, and 5 units of Taq enzyme (Promega Co). Dideoxynucleotide termination sequencing was performed according to the manufacturer's instructions. Cycle sequencing was performed for 30 rounds. Denaturation, annealing and extension steps were for 30 seconds, 30 seconds and 1 minute, respectively.

#### 15 Cloning and Sequencing of S Gene

Direct sequencing results showed that the first serum from patient two (February 1993) had evidence of a mixture of wild-type and mutant viruses. PCR products from this serum sample and subsequent ones from patient No. 2 were cloned into the pUC19 vector to confirm direct sequencing results. Nucleotide sequences of amplified HBV-DNA were determined with the sequenase DNA sequencing kit (Version 2.0, USB, Cambridge, England).

#### 25 Hydrophobicity Plot

Amino acids 122 to 137 and 122 to 139 or 140 (including inserted amino acids) of the 'a' determinant of HBsAg from the wild type and variant viral isolates, respectively, were analysed with the Prosis software (Pharmacia, St. Albans, England).

#### Dot Blot Hybridisation

HBV-DNA was detectable in serum from Patient No. 1 following amplification by PCR on two separate occasions.



In Patient No. 2, this was detectable by dot blot hybridisation in March 1993 and September 1993, at 10  $\mu\text{g}/50 \mu\text{L}$  of serum. Given that 1.0  $\mu\text{g}$  of HBV-DNA is equivalent to  $2.3 \times 10^6$  HBV genomes, it was estimated that this patient's serum contained about  $4.6 \times 10^7$  viral particles per mL. HBsAg was detectable in the first but not the second sample. In January 1994, HBV-DNA was detectable only by PCR, and HBsAg was still undetectable.

#### 10 Monoclonal Antibody Binding Studies

Binding studies in Patient No. 1 and No. 2 and an HBsAg positive patient used as a control (adw), showed that the HBsAg in the serum bound to all three monoclonal antibodies in Patient 2 and could be detected by the polyclonal anti-HBs from the AusRIA II kit (Table 2).

#### Sequencing of the Pre S-S Gene and Pre C/C Region

The S genes consist of 687 bp in Isolate 1, 690 in Isolate 2, respectively, and 681 bp in the wild types. The S nucleotide and amino acid sequences of the mutants were compared with a published sequence of the same subtype (adw), and also with a wild type strain from an HBeAg-positive carrier from the same region (as shown in Table 3).

Sequencing results revealed an insertion in the S gene (Figure 4). Inserted sequences encode two additional amino acids (Arg-Ala) between codons 122 and 123 in Isolate 1, and three additional amino acids (Arg-Gly-Ala) between codons 123 and 124 in Isolate 2 (Fig. 5). These insertions occur immediately before the 'a' determinant of HBsAg. Such insertions have not been described in published sequences of known HBV subtypes and were absent from consensus sequences obtained from 30 Chinese HBsAg positive patients from the same region of China.

Direct sequencing results were verified by cloning sequencing. Of 10 clones from the first serum taken from Patient No. 2, 8 had an in-phase insertion as described above, 2 of them were wild type, whereas all 15 clones from the second and third serum samples were of the variant. Thus, the first serum from Patient No. 2 had evidence of a mixture of wild-type and mutant viruses, indicating gradual emergence of the mutant, which in subsequent sera became the predominant species.

There were no previously described amino acid deletions or substitutions in the pre-S1, pre-S2 genes.

#### Hydrophobic Plot

This was done according to the method of Kyte and Doolittle, giving a mean hydrophobicity index of -0.48 for amino acids 122-137 of the wild type 'a' determinant -0 for amino acids 122-139 in isolate 1 and -0.89 for amino acids 122-140 in isolate 2. Thus, the mutant 'a' determinants were more hydrophobic than the same region from the wild-type virus.

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**PATENTS**

- European Patent No. 0226846  
European Patent No. 0278940  
10 European Patent No. 0299108  
PCT Application No. 91/14703  
PCT Application No. 94/26904  
U.S. Patent 4,649,192  
U.S. Patent 4,683,294  
15 U.S. Patent 4,694,074  
U.S. Patent 4,738,926

CLAIMS

1. A hepatitis B surface antigen protein wherein said protein displays the antigenicity of HBV surface antigen, characterised in that said protein (mHBsAg) comprises an antigenically modified envelope region in which there are at least two amino acids inserted after codon position 122 of the HBsAg sequence.
2. A mHBsAg as claimed in claim 1 in which the amino acid residues inserted after position 122 are such as to reduce the hydrophilicity of the envelope region of HBV.
3. A mHBsAg as claimed in claim 1 or claim 2 in which the codons inserted after position 122 of normal HBsAg encode arginine and alanine, in that order.
4. A mHBsAg as claimed in claim 1 or claim 2 in which the codons inserted after position 122 of normal HBsAg encode arginine, glycine and alanine, in that order.
5. A mHBsAg as claimed in any one of claims 1 to 4 which is identical to normal HBsAg S-protein except for the insertion of at least two amino acid residues after position 122.
6. A mHBsAg as claimed in any one of claims 1 to 5 in which the insertion after position 122 is arginine and alanine, in that order.
7. A mHBsAg as claimed in any one of claims 1 to 5 in which the insertion after position 122 is arginine, glycine and alanine, in that order.

8. A mHBsAg as claimed in any one of claims 1 to 7 in which the mHBsAg include Pre-S sequences.

5 9. A mHBsAg as claimed in any one of claims 1 to 8 which is greater than 75% pure.

10. An expression vector comprising a DNA sequence encoding a mHBsAg according to any one of claims 1 to 8.

10 11. A host transformed with the vector of claim 10.

12. A vaccine composition comprising an effective amount of a mHBsAg as claimed in claim 9, admixed with a suitable carrier or adjuvant.

15 13. A process for preparing a mHBsAg as claimed in any one of claims 1 to 9, which process comprises the steps of culturing a host according to claim 11 in an appropriate culture medium, and purifying the mHBsAg  
20 produced to the required degree of purity.

14. The use of a mHBsAg according to any one of claims 1 to 9 in the manufacture of a vaccine for protecting a human against disease associated with HBV.

25 15. A kit for diagnostic *in vitro* detection of anti-mHBsAg antibodies in a biological medium, characterised in that the kit comprises:

30 (a) mHBsAg according to any one of claims 1 to 8; and  
(b) means adapted to detect the antigen-antibody reaction.

16. An antibody preparation comprising antibodies against mHBsAg, as defined in any one of claims 1 to 9 for use in the diagnosis, prevention or treatment of hepatitis B infection in humans.

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FIG. 1

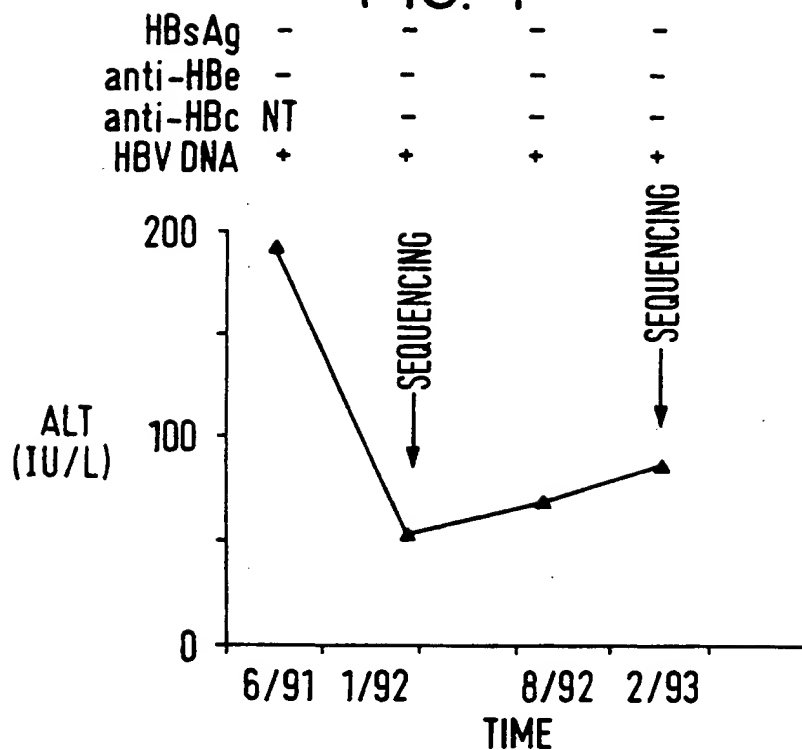
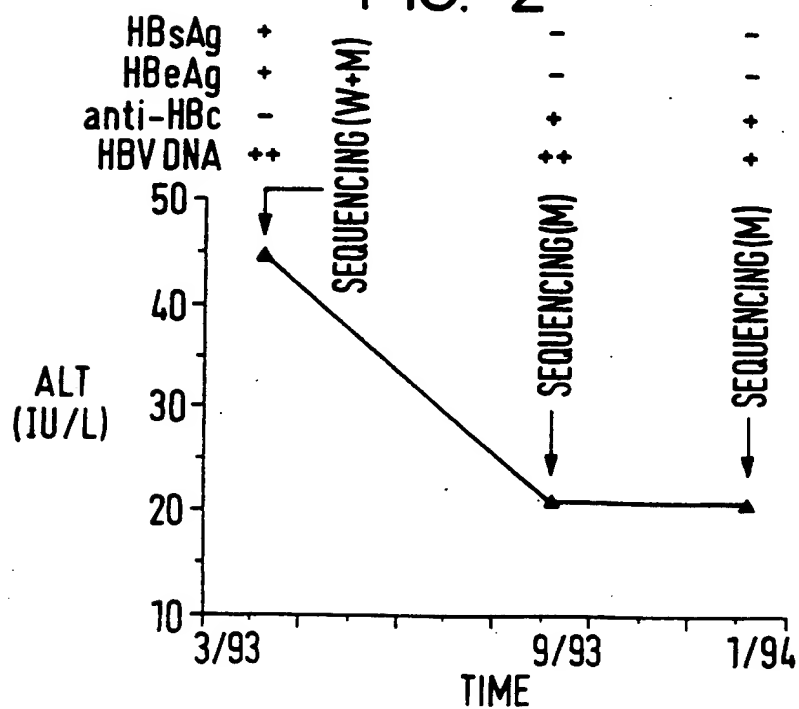


FIG. 2

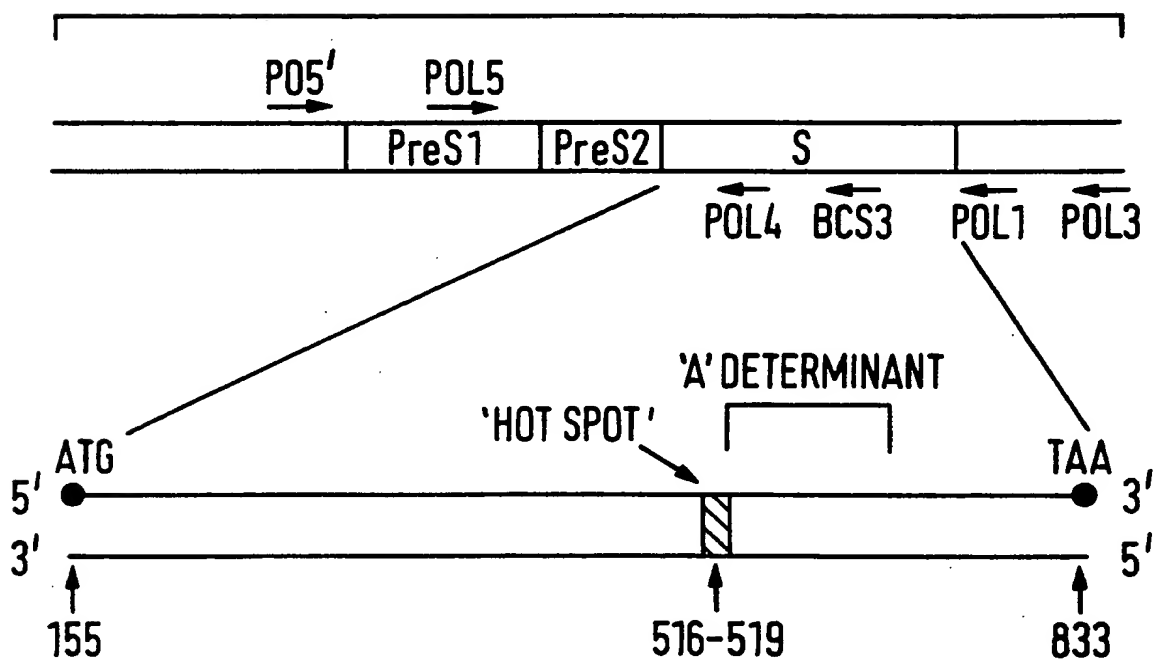




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FIG. 3

P ORF



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FIG. 4

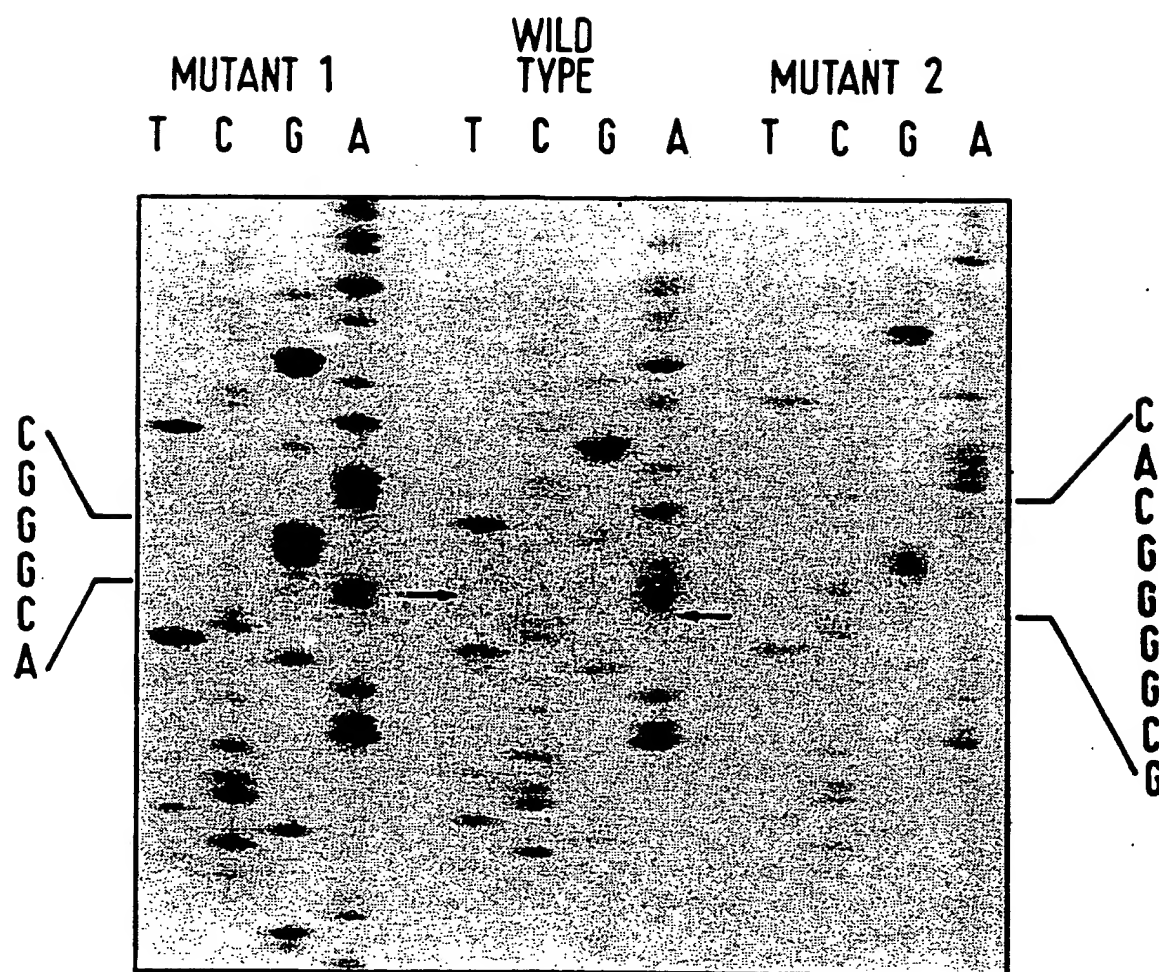


FIG. 5

	514		521		532	
	↓		↓		↓	
WILD TYPE	T G C A A A	C C T G C A C A A C T C C T				4 / 7
(adw)	121 122	123 124 125 126 127				
	Cyz Lys	Thr Cys Thr Thr Thr Pro				
MUTANT 1	- - - - - C G G G C A	A - - - - -				
	- - - - - Arg Ala	- - - - -				
MUTANT 2	- - - - - C A C G G G C G	- - - - -				
	- - - - - Thr Arg Gly Ala	- - - - -				

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FIG. 6

10 20 30 40 50 60  
ATGGAGAACATCACATCAGGACTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTC  
X:::  
ATGGAGAACATCGCATCAGGACTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTC  
160 170 180 190 200 210  
70 80 90 100 110 120  
TTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAAT  
:::  
TTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAAT  
220 230 240 250 260 270  
130 140 150 160 170 180  
TTTCTAGGGGGAACACCCGTGTGTCTTGGCCAAAATTTCGCAGTCCCAAATCTCCAGTCAC  
:::  
TTTCTAGGGGGAACACCCGTGTGTCTTGGCCAAAATTTCGCAGTCCCAAATCTCCAGTCAC  
280 290 300 310 320 330  
190 200 210 220 230 240  
TCACCAACCTGTTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTT  
:::  
TCACCAACTTGTGTCCTCCGATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTT  
340 350 360 370 380 390  
250 260 270 280 290 300  
ATCATCTTCCTCTGCATCCTGCTGCTATGCCTCATCTTCTTGTGGTTCTTCTGGACTAT  
:::  
ATCATCTTCCTCTGCATCCTGCTGCTATGCCTCATCTTCTTGTGGTTCTTCTGGACTAT  
400 410 420 430 440 450  
310 320 330 340 350 360  
CAAGGTATGTTGCCCGTTTGTCTCTAATTCCAGGATCATCAACAACCAGCACCGGACCA  
:::  
CAAGGTATGTTGCCCGTTTGTCTCTAATTCCAGGATCATCAACCAGCACCGGACCA  
460 470 480 490 500 510  
370 380 390 400 410 420  
TGCAAAACGGGCAACCTGCACAACCTCCTGCTCAAGGAACCTCTATGTTTCCCTCATGTTGC  
:::::X :::  
TGCAAA-----ACCTGCACGACTCCTGCTCAAGGAACCTCTATGTTTCCCTCATGTTGC  
520 530 540 550 560  
430 440 450 460 470 480  
TGTACAAAACCTACGGACGGAAACTGCACCTGTATTCCCATCCCATCATCTTGGCGTTTT  
:::  
TGTACAAAACCTACGGACGGAAACTGCACCTGTATTCCCATCCCATCATCTTGGCGTTTT  
570 580 590 600 610 620  
490 500 510 520 530 540  
GCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCA  
:::  
GCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCA  
630 640 650 660 670 680  
550 560 570 580 590 600  
TTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTCTGGCTTTCAGTTATATGGATGATG  
:::  
TTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTCTGGCTTTCAGTTATATGGATGATG  
690 700 710 720 730 740  
610 620 630 640 650 660  
TGGTTTTGGGGCCAAGTCTGATCAACATCTTGAGTCCCTTTATGCCGCTGTTACCAATT  
:::  
TGGTTTTGGGGCCAAGTCTGATCAACATCTTGAGTCCCTTTATGCCGCTGTTACCAATT  
750 760 770 780 790 800  
670 680  
TTCTTTTGTCTTTGGGTATACATTAA  
:::  
TTCTTTTGTCTTTGGGTATACATTAA  
810 820 830

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FIG. 7

```

      10      20      30      40      50      60
ATGGAGAACATCACATCAGGACTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTC
X:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
ATGGAGAACATCGCATCAGGACTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTC
      10      20      30      40      50      60
      70      80      90     100     110     120
TTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAAT
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
TTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAAT
      70      80      90     100     110     120
      130     140     150     160     170     180
TTTCTAGGGGGAACACCCGTGTGTCTTGGCCAAAATTTCGCAGTCCCAAATCTCCAGTCAC
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
TTTCTAGGGGGAACACCCGTGTGTCTTGGCCAAAATTTCGCAGTCCCAAATCTCCAGTCAC
      130     140     150     160     170     180
      190     200     210     220     230     240
TCACCAACCTGTTGTCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTT
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
TCACCAACTTGTTGTCTCCGATTGTCTCTGGTTATCGCTGGATGTGTCTGCGGCGTTTT
      190     200     210     220     230     240
      250     260     270     280     290     300
ATCATCTTCTCTGCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTAT
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
ATCATCTTCTCTGCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTAT
      250     260     270     280     290     300
      310     320     330     340     350     360
CAAGGTATGTTGCCCCGTTTGTCTCTAATTCCAGGATCATCAACAACCAGCACCGGACCA
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
CAAGGTATGTTGCCCCGTTTGTCTCTAATTCCAGGATCATCAACCACCAGCACCGGACCA
      310     320     330     340     350     360
      370     380     390     400     410     420
TGCAAAACACGGGGCGCCTGCACAACCTCTGCTCAAGGAACCTCTATGTTTCCCTCATGT
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
TGCAAAA-----CCTGCACGACTCCTGCTCAAGGAACCTCTATGTTTCCCTCATGT
      370     380     390     400     410
      430     440     450     460     470     480
TGCTGTACAAAACCTACGGACGGAAACTGCACCTGTATTCCCATCCCATCATCTTGGGCT
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
TGCTGTACAAAACCTACGGACGGAAACTGCACCTGTATTCCCATCCCATCATCTTGGGCT
      420     430     440     450     460     470
      490     500     510     520     530     540
TTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTG
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
TTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTG
      480     490     500     510     520     530
      550     560     570     580     590     600
CCATTTGTTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTCTGGCTTTCAGTTATATGGATG
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
CCATTTGTTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTCTGGCTTTCAGTTATATGGATG
      540     550     560     570     580     590
      610     620     630     640     650     660
ATGTGGTTTTGGGGGCAAGTCTGTACAACATCTTGAGTCCCTTTATGCCGCTGTTACCA
:::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
ATGTGGTTTTGGGGGCAAGTCTGTACAACATCTTGAGTCCCTTTATGCCGCTGTTACCA
      600     610     620     630     640     650
      670     680     690
ATTTTCTTTTGTCTTTGGGTATACATTTAA
:::::::::::: ::::::::::::::^::::::::::v
ATTTTCTTTTGTCTTTGGGTATACATTTAA
      660     670     680

```

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TABLE 1

DESIGNATION			SEQUENCES	NUCLEOTIDE POSITION	
Pre-S1	PO5 <sup>1</sup>	ANTISENSE	5 <sup>1</sup> -TGCGGGTCACCATAT	(2818-2833)	
Pre-S2	POL5	ANTISENSE	5 <sup>1</sup> -CAATCGGCAGTCAGG	(3125-3139)	
	S {	POL4	SENSE	5 <sup>1</sup> -AGGCATAGCAGCAGG	(428-414)
		BCS3	SENSE	5 <sup>1</sup> -GGCACTAGTAAACTGAGCCA	(687-660) <sup>1</sup>
		POL1	SENSE	5 <sup>1</sup> -GCAAAGTTCCC CAACTTC	(912-895)
		POL3	SENSE	5 <sup>1</sup> -AAGGATCCAGTTGGC	(1409-1395)

<sup>1</sup>CARMAN et al., 1990

TABLE 2

	DATE	RFHBs1	RFHBs2	RFHBs7	AusRIA
PATIENT 1	FEBRUARY 1993	-1	-	-	-
PATIENT 2	SEPTEMBER 1993	31	23	2	-
CONTROL		19	176	3	74

1 NEGATIVE

TABLE 3

	WILD TYPE adw		CHINESE adw	
	nt %	aa %	nt %	aa %
ISOLATE 1	94.0	91.2	93.7	90.8
ISOLATE 2	93.6	90.8	93.3	90.4

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB95/00208A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/02 A61K39/29 G01N33/576

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO,A,94 26904 (ABBOTT LABORATORIES ET AL.) 24 November 1994 cited in the application see page 6, line 5 - page 8, line 19 ---	1
A	EP,A,0 511 855 (MERCK & CO. INC.) 4 November 1992 see page 6, line 23 - page 11, line 40 -----	1

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Date of the actual completion of the international search

16 May 1995

Date of mailing of the international search report

31-05-1995

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No

PCT/GB 95/00208

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9426904	24-11-94	AU-B- 6908294	12-12-94
EP-A-0511855	04-11-92	JP-A- 6172393	21-06-94